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Nucleosomes occurring in protein-free hybridoma cell culture

Evidence for programmed cell death

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In addition to monoclonal immunoglobulin, two kinds of nucleoproteins, NP1 and NP2, were isolated from the supernatants of hybridoma cultures set up in a protein-free medium. As shown by SDS-electrophoresis the two nucleoproteins shared a set of proteins (apparent M_r 11000 to 15000), and differed in the DNA moiety (~150 bp in NP1, ~350 bp in NP2). The amino acid composition of the protein moiety confirmed the nucleosomal origin of NP1 and NP2. The findings support the view that in hybridoma cultures the cells undergo death by apoptosis, i.e. a programmed process characterized by initial fragmentation of chromatin.

Nucleosome; Chromatin; B hybridoma; Cell culture; Cell death; Apoptosis

1. INTRODUCTION

The growth of cultured hybridoma cells and the secretion of monoclonal antibody are dependent on the presence of both external and autocrine factors. Serum proteins used according to a formerly accepted view as indispensable external supplements to the medium [1,2] could introduce into the system hardly detectable trace impurities with unknown activities. Moreover, the proteins interfered with the fine analysis of substances secreted by the cells. The protein-free medium developed recently in this laboratory [3] overcame entirely the necessity of supplementing the medium with external macromolecular substances. When analysing the hybridoma culture fluid devoid of background external macromolecules we observed that the monoclonal immunoglobulin was accompanied by a relatively large quantity of nucleoproteins. In this paper we present evidence that the source of the nucleoproteins is degraded chromatin, and we suggest a biological mechanism underlying this phenomenon.

2. MATERIAL AND METHODS

2.1. *Cell culture*

The characteristics of mouse hybridomas used in this work were the following: TSH-5.07 (fusion partner Sp2/O-Ag14), IgG_1 antibody to human thyrotropin; CMH-01 (fusion partner FO)), IgG_1 antibody reacting with a group of steroid substances; LNKB (fusion partner X63-Ag8.653), IgG_1 antibody to IL-2 (gift of Dr. V. Nesmeyanov, Institute of Bioorganic Chemistry, Moscow).

The cells were cultured in a mixture of basal media RPMI-1640 and DMEM (1:1) enriched in glucose to 4 g/l, and supplemented with glutamine (300 mg/l), HEPES (15 mM), antibiotics, and with the following growth-stimulating substances: ferric citrate (500 μM), ethanolamine (20 μM), ascorbic acid (20 μM), hydrocortisone (5 nM), and salts of several trace elements [3]. The cells grown in spinner flasks were inoculated into a stirred laboratory bioreactor (LF-2, Workshops of the Czechoslovak Academy of Science) with a working volume of 1.2–1.8 liters. The bioreactor was equipped with an automatic control of pH (7.2–7.3) and dissolved oxygen concentration (30% air saturation). Batch cultures grown for 4–6 days reached total cell densities in the range of 1.2 to 2.2 × 10^6 cells/ml, as counted in a Bürker cytometer.

2.2. *Processing of the culture fluid*

Clear supernatant was supplemented with sodium citrate (final concentration 100 mM), concentrated by ultrafiltration (Amicon PM-30 membrane) to 1/10 of the original volume and applied to a column of Sephadex G-50. The material from the break-through peak was collected and subjected to further separation steps, i.e. gel chromatography on Sephacryl S-300 HR ($NaCl/P_i$), and gel chromatography on Biogel P-100 (4 M guanidinium hydrochloride).

2.3. *Analytical methods*

SDS/polyacrylamide gel electrophoresis was carried out using a 0.1% SDS/12% acrylamide/0.32% bisacrylamide gel. The amino acid composition was determined on a Durrum D-500 automatic amino acid analyzer. The absorption spectra were recorded on a spectrophotometer (Shimadzu UV-260). The content of DNA was estimated using the coefficient A_{260} (1 mg/ml) = 20.0. The sedimentation coefficient was determined on an analytical ultracentrifuge (Spinco Model E).

3. RESULTS

3.1. *Separation of the culture fluid components*

The culture fluid of the model hybridoma TSH-5.07 was freed of low-molecular weight components by gel

Abbreviations: $NaCl/P_i$, phosphate-buffered saline, pH 7.4

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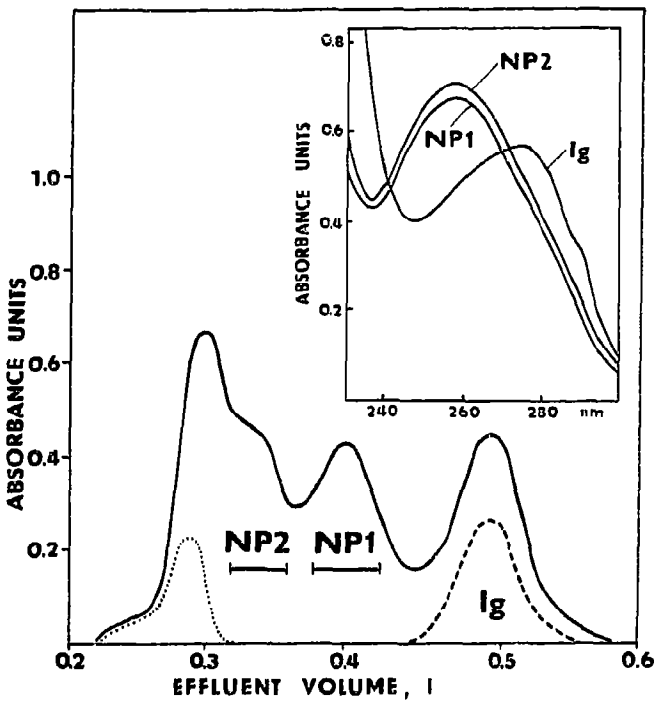

Fig. 1. Gel chromatography of the macromolecular portion of the TSH-5.07 hybridoma culture fluid. The column of Sephacryl S-300 HR (5.3 × 47 cm) was equilibrated and run in NaCl/P_i. The presence of monoclonal immunoglobulin was estimated by a turbidimetric immunoassay using porcine antiserum against mouse IgG_1. Full line = A_{280}; dotted line = turbidity (A_{550} × 10); dashed line = turbidity with anti-Ig serum (A_{550}). (Inset) Absorption spectra of indicated pooled fractions.

filtration on Sephadex G-50. Sephadex G-25 was found to be less suitable for this purpose, because the ferric ion in the culture medium was obviously incorporated into complexes possessing a larger molecular size than that corresponding to the formula weight of ferric citrate. The slightly turbid colorless solution of the high-molecular weight components of the culture fluid was applied to the column of Sephacryl S-300 HR. The elution profile showed several peaks, one of which could be identified by immunoassay as immunoglobulin (Fig. 1). The spectra of clear fractions emerging between the turbid break-through portion and the immunoglobulin peak displayed absorption maxima near 260 nm (Fig. 1, inset).

3.2. *Characteristics of the nucleoproteins*

The electrophoretic patterns of the material contained in the peaks NP1 and NP2 showed the presence of a set of bands stained both with Coomassie blue and silver. A single additional band (NP1) or one major and a minor band (NP2) were stained solely with silver (Fig. 2). The NP1 and NP2 nucleoproteins were digested by pancreatic DNase or by pancreatic RNase. The silver-stained bands disappeared upon DNase digestion, but remained intact upon RNase treatment (not shown).

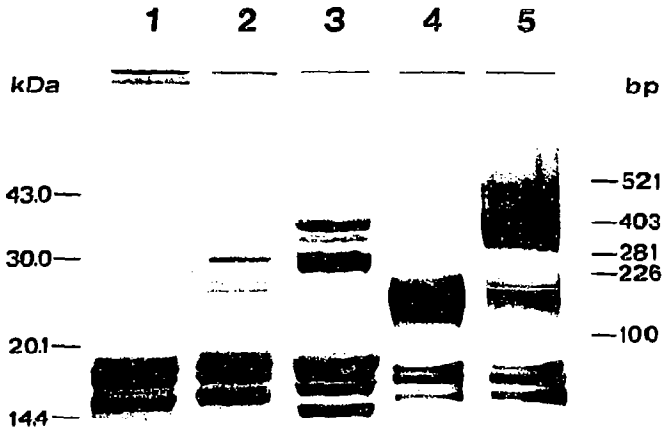

Fig. 2. Electrophoretic analysis of nucleoproteins NP1 and NP2. SDS-polyacrylamide gel (12%) system. Lanes 1–3 were stained with 0.3% Coomassie blue, lanes 4 and 5 with silver [4]. Left margin = protein calibration mixture (Pharmacia); right margin = DNA calibration mixture (pBR/Alu). (Lanes 1 and 4) NP1; (lanes 2 and 5) NP2, (lane 3) histone standards.

The apparent M_r values of the protein bands corresponded to those of the histones forming the nucleosome core, i.e. 11 000 to 15 000. The size of the silver-stained bands, ~150 bp and ~350 bp, corresponded to the length of DNA segments constituting a mononucleosome or a dinucleosome, respectively. The sedimentation coefficient of the NP1 nucleoprotein, $s_{20,w}$ = 10.8 S, was in agreement with the sedimentation coefficient of mononucleosomes [5].

3.3. *Characteristics of the protein moiety*

The apparent molecular masses and the general pattern in SDS electrophoresis pointed to the identity of the protein moiety of NP1 and NP2 with core histones. In order to strengthen this evidence, the proteins of NP1 were separated from DNA by Biogel-100 chromatography. Dissociated proteins could be collected as a retarded peak. The amino acid composition of these proteins was compared with the theoretical composition of the histone octamer [6–8] (Table I). The histone nature of the protein moiety of NP1 was evident from the fairly good agreement with the theoretical values of amino acids of typically low occurrence (cysteine, methionine, histidine), of typically high occurrence (lysine, arginine), and of hydrophobic amino acids (valine, leucine, isoleucine). However, the content of aspartic acid, glycine and alanine deviated from the theory to a degree that could hardly be ascribed to experimental error. The presence of variant histones or of a non-stoichiometric admixture of non-histone chromatin components could not be ruled out.

3.4. *Occurrence of nucleosomes in various hybridoma cultures*

In addition to the main model hybridoma TSH-5.07, the hybridomas CHM-01 and LNKB were found to

Table I

Amino acid composition of the total protein isolated from the nucleoprotein NP1

Amino acid	Total protein[a]	Histone octamer[b]	Difference[c]
	(Residues per 1000)		(%)
Lysine	111.5	116.0	−3.9
Histidine	23.1	22.4	+3.1
Arginine	105.5	107.8	−2.1
Cysteine[d]	3.9	4.1	−4.9
Aspartic acid	60.9	48.9	+24.5
Threonine	62.0	61.1	+1.5
Serine	57.6	53.0	+8.1
Glutamic acid	92.6	87.6	+5.7
Proline	33.6	36.7	−8.4
Glycine	81.1	91.7	−11.6
Alanine	96.6	110.0	−12.2
Valine	69.3	63.1	+9.8
Methionine	11.0	10.2	+7.8
Isoleucine	51.4	50.9	+1.0
Leucine	86.4	87.6	−1.4
Tyrosine	33.6	30.6	+9.8
Phenylalanine	19.9	18.3	+8.7

[a]Mean of 4 independent analyses. The samples were hydrolyzed in 6 M hydrochloric acid for 20 and 70 h at 110°C.
[b]Composition of mouse histone octamer derived from amino acid sequence data based on gene sequencing: H2A [6], H2B, H3.1 [7], H4 [8].
[c]Difference between value found and the theoretical value expressed as percent of the theoretical value.
[d]Determined as cysteic acid in an oxidized aliquot.

liberate nucleoproteins NP1 and NP2 into their culture fluids (Table II). The concentration of the nucleosomes increased with the time of duration of the culture. The total concentration of nucleosomes might be assessed when summing the yields of DNA obtained from NP1 and NP2, multiplying this by a factor, e.g. 1.5 that would compensate for the losses occurring upon isolation, and adding the mass of histone octamers. A rough estimate obtained in this way was 10–20 μg/ml, i.e. a mass concentration of the same order of magnitude as was the concentration of secreted immunoglobulin.

4. DISCUSSION

The nucleoproteins found in the hybridoma culture fluid share their principal characteristics with nuclease resistant chromatin fragments described earlier by Sarasbuhde and VanHolde [5]. The presence of free nucleosomes in the culture fluid indicated that an intense activation of endonucleases had to precede their liberation, and that the integrity of some cells had to be lost. These phenomena are typical for cell death by apoptosis [9], i.e. programmed cell death different from necrosis. The salient feature of apoptosis is the initial degradation of chromatin.

In cultured T cells [10] or in T-cell hybridomas [11], the programmed cell death was found to occur as a consequence of the cell activation process. In the cultures of B-cell hybridomas, i.e. cultures of the cell type investigated in this work, apoptosis has been suggested by an electron microscopic study [12]. The findings of our present work based on biochemical analysis add substantial weight to the view, that in hybridoma cultures at least a fraction of cells dies via apoptosis. Demonstration of fragmented chromatin inside the cells as well as of insoluble cell fragments cross-linked by transglutaminase [13] is currently in progress.

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Table II

Yield of DNA from hybridoma culture fields

Hybridoma clone	Cell count (cells/ml × 10^{-6})	Viability[a] (%)	Yield of DNA obtained from nucleoproteins (μg/ml)		
			NP1	NP2	NP1 + NP2
TSH-5.07	0.85	91	0.98	0.69	1.67
	2.24	80	2.20	2.52	4.72
CMH-01	1.37	77	2.28	2.10	4.38
LNKB	1.11	63	1.63	0.86	2.49

[a]Trypan blue exclusion test.